

**HYPERSENSITIVE RESPONSE ELICITOR
FROM *ERWINIA AMYLOVORA* AND ITS USE**

5 This application claims benefit of U.S. Provisional Patent Application
Serial No. 60/055,108, filed August 6, 1997.

FIELD OF THE INVENTION

10 The present invention relates to a hypersensitive response elicitor from
Erwinia amylovora and its use.

BACKGROUND OF THE INVENTION

15 Interactions between bacterial pathogens and their plant hosts generally
fall into two categories: (1) compatible (pathogen-host), leading to intercellular
bacterial growth, symptom development, and disease development in the host plant;
and (2) incompatible (pathogen-nonhost), resulting in the hypersensitive response, a
particular type of incompatible interaction occurring, without progressive disease
20 increase dramatically and progressive symptoms occur. During incompatible
interactions, bacterial populations do not increase, and progressive symptoms do not
occur.

The hypersensitive response is a rapid, localized necrosis that is
associated with the active defense of plants against many pathogens (Kiraly, Z.,
25 "Defenses Triggered by the Invader: Hypersensitivity," pages 201-224 in: Plant
Disease: An Advanced Treatise, Vol. 5, J.G. Horsfall and E.B. Cowling, ed.
Academic Press New York (1980); Klement, Z., "Hypersensitivity," pages 149-177
in: Phytopathogenic Prokaryotes, Vol. 2, M.S. Mount and G.H. Lacy, ed. Academic
Press, New York (1982)). The hypersensitive response elicited by bacteria is readily
30 observed as a tissue collapse if high concentrations ($\geq 10^7$ cells/ml) of a limited
host-range pathogen like *Pseudomonas syringae* or *Erwinia amylovora* are infiltrated
into the leaves of nonhost plants (necrosis occurs only in isolated plant cells at lower
levels of inoculum) (Klement, Z., "Rapid Detection of Pathogenicity of
Phytopathogenic Pseudomonads," Nature 199:299-300; Klement, et al.,

“Hypersensitive Reaction Induced by Phytopathogenic Bacteria in the Tobacco Leaf,” Phytopathology 54:474-477 (1963); Turner, et al., “The Quantitative Relation Between Plant and Bacterial Cells Involved in the Hypersensitive Reaction,” Phytopathology 64:885-890 (1974); Klement, Z., “Hypersensitivity,” pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York (1982)). The capacities to elicit the hypersensitive response in a nonhost and be pathogenic in a host appear linked. As noted by Klement, Z., “Hypersensitivity,” pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York, these pathogens also cause physiologically similar, albeit delayed, necroses in their interactions with compatible hosts. Furthermore, the ability to produce the hypersensitive response or pathogenesis is dependent on a common set of genes, denoted *hrp* (Lindgren, P.B., et al., “Gene Cluster of *Pseudomonas syringae* pv. ‘phaseolicola’ Controls Pathogenicity of Bean Plants and Hypersensitivity on Nonhost Plants,” J. Bacteriol. 168:512-22 (1986); Willis, D.K., et al., “*hrp* Genes of Phytopathogenic Bacteria,” Mol. Plant-Microbe Interact. 4:132-138 (1991)). Consequently, the hypersensitive response may hold clues to both the nature of plant defense and the basis for bacterial pathogenicity.

The *hrp* genes are widespread in gram-negative plant pathogens, where they are clustered, conserved, and in some cases interchangeable (Willis, D.K., et al., “*hrp* Genes of Phytopathogenic Bacteria,” Mol. Plant-Microbe Interact. 4:132-138 (1991); Bonas, U., “*hrp* Genes of Phytopathogenic Bacteria,” pages 79-98 in: Current Topics in Microbiology and Immunology: Bacterial Pathogenesis of Plants and Animals - Molecular and Cellular Mechanisms, J.L. Dangel, ed. Springer-Verlag, Berlin (1994)). Several *hrp* genes encode components of a protein secretion pathway similar to one used by *Yersinia*, *Shigella*, and *Salmonella* spp. to secrete proteins essential in animal diseases (Van Gijsegem, et al., “Evolutionary Conservation of Pathogenicity Determinants Among Plant and Animal Pathogenic Bacteria,” Trends Microbiol. 1:175-180 (1993)). In *E. amylovora*, *P. syringae*, and *P. solanacearum*, *hrp* genes have been shown to control the production and secretion of glycine-rich, protein elicitors of the hypersensitive response (He, S.Y., et al. “*Pseudomonas Syringae* pv. *Syringae* HarpinPss: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants,” Cell 73:1255-1266 (1993), Wei, Z.-H.,

et al., "HrpI of *Erwinia amylovora* Functions in Secretion of Harpin and is a Member of a New Protein Family," J. Bacteriol. 175:7958-7967 (1993); Arlat, M. et al.

"PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-553 (1994)).

The first of these proteins was discovered in *E. amylovora* Ea321, a bacterium that causes fire blight of rosaceous plants, and was designated harpin (Wei, Z.-M., et al., "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992)). Mutations in the encoding *hrpN* gene revealed that harpin is required for *E. amylovora* to elicit a hypersensitive response in nonhost tobacco leaves and incite disease symptoms in highly susceptible pear fruit. The *P. solanacearum* GMI1000 PopA1 protein has similar physical properties and also elicits the hypersensitive response in leaves of tobacco, which is not a host of that strain (Arlat, et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-53 (1994)). However, *P. solanacearum popA* mutants still elicit the hypersensitive response in tobacco and incite disease in tomato. Thus, the role of these glycine-rich hypersensitive response elicitors can vary widely among gram-negative plant pathogens.

Other plant pathogenic hypersensitive response elicitors have been isolated, cloned, and sequenced. These include: *Erwinia chrysanthemi* (Bauer, et. al., "*Erwinia chrysanthemi* Harpin_{Ech}: Soft-Rot Pathogenesis," MPMI 8(4): 484-91 (1995)); *Erwinia carotovora* (Cui, et. al., "The RsmA⁻ Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrpN*_{Ecc} and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI 9(7): 565-73 (1996)); *Erwinia stewartii* (Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microb. Inter. July 14-19, 1996 and Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," Ann. Mtg. Am. Phytopath. Soc. July 27-31, 1996); and *Pseudomonas syringae* pv. *syringae* (WO 94/26782 to Cornell Research Foundation, Inc.).

The present invention is a further advance in the effort to identify, clone, and sequence hypersensitive response elicitor proteins or polypeptides from plant pathogens.

5

SUMMARY OF THE INVENTION

The present invention is directed to an isolated protein or polypeptide which elicits a hypersensitive response in plants as well as an isolated DNA molecule which encodes the hypersensitive response eliciting protein or polypeptide.

10

The hypersensitive response eliciting protein or polypeptide can be used to impart disease resistance to plants, to enhance plant growth, and/or to control insects. This involves applying the hypersensitive response elicitor protein or polypeptide in a non-infectious form to plants or plant seeds under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds.

15

As an alternative to applying the hypersensitive response elicitor protein or polypeptide to plants or plant seeds in order to impart disease resistance, to enhance plant growth, and/or to control insects on plants, transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding a hypersensitive response elicitor protein or polypeptide and growing the plant under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects in the plants or plants grown from the plant seeds. Alternatively, a transgenic plant seed transformed with the DNA molecule encoding a hypersensitive response elicitor protein or polypeptide can be provided and planted in soil. A plant is then propagated under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds.

20

25

BRIEF DESCRIPTION OF THE DRAWINGS

30

Figures 1 A and B show the molecular structure of the region of *E. amylovora* genome containing *hrpW*. Figure 1A depicts cosmids pCPP430 and pCPP 450 that contain the regulatory and secretory region of the *hrp* cluster of *E*.

amylovora. Arrow boxes on top of the cosmid clones indicate the transcriptional units, where the names of the characterized operons are given above (Wei, et al., Science, 257:85-88 (1992); Zumoff, et al., The hrp Gene Cluster of Erwinia amylovora, eds. Hennecke, H. & Verma, D.P.S. (Kluwer Academic Publishers,

- 5 Dordrecht, The Netherlands), Vol. 1, pp. 53-60 (1991); Bogdanove, et al., J. Bacteriol., 178:1720-30 (1996); and Kim, et al., J. Bacteriol., 179:1690-97 (1997), which are hereby incorporated by reference). Figure 1B shows the location of *hrpW* which encodes a Gly-rich protein, and subclones of pCPP1012 used in the study. Boxes and arrow boxes indicate genes or open reading frames; filled triangles are
10 putative HrpL-dependent promoters. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III, Ea, *Eag*I; Hp, *Hpa*I.

Figure 2A shows the expression of *hrpW* by a T7 RNA polymerase-directed gene expression system. Lanes 1, *E. coli*DH5 α (pGP1-2/pBC^{SK} (-)); 2, *E. coli*DH5 α (pGP1-2/pCPP1232). The arrow between 84kD and 53kD points to the
15 band in lane 2 corresponding to the HrpW protein.

sub
DI
7
Figure 3 shows the alignment of HrpW with pectate lyases of *Nectria haematococca*, mating type VI (*Fusarium solani* f. sp. *pisi*) and of *Erwinia carotovora* subsp. *carotovora*. The sequences were aligned by the PILEUP program (GCG software package, Version 7.3) with default parameters, and an alignment was
20 manually edited using LINEUP program in the same package. Conserved residues are boxed, highly conserved regions are underlined, and potential α -helices in HrpW are shaded.

- Figures 4A and B are immunoblots showing the *hrp*-dependent production and secretion of HrpW in *E. amylovora*. Lanes in Figure 4: 1, *E. coli*
25 DH5 α (pGP1-2/pCPP1232); 2, HrpN; 3, whole cell preparation ("CP") of Ea321; 4, supernatant preparation ("SP") of Ea321; 5, CP of Ea321-K49; 6, SP of Ea321-K49; 7, CP of Ea321-G84; 8, SP of Ea321-G84. Lanes in Figure 4B: 1, *E. coli* DH5 α (pGP1-2/pCPP1232); 2, HrpN; 3, CP of Ea273; 4, SP of Ea273; 5, CP of Ea321-K49; 6, SP of Ea321-K49; 7, CP of Ea321-G73; 8, SP of Ea321-G73.

- 30 Figure 5A shows a tobacco leaf showing residual hypersensitive response ("HR") eliciting activity of *hrpN* mutants, and the HR induced by HrpN and HrpW. Panels: 1, *E. coli*DH5(pCPP430); 2, *E. coli*DH5(pCPP430-T5); 3, *E.*

*coli*MC4100(pCPP450); 4, *E. coli*MC4100(pCPP450-T5); 5, 5 mM KPO₄ buffer (pH 6.5); 6, *E. amylovora* Ea321; 7, *E. amylovora* Ea321-T5; 8, HrpN CFEP (contains 0.5 mg/ml of HrpN); 9, HrpW preparation (0.5 mg/ml) eluted from the gel containing proteins from *E. coli*DH5α(pGP1-2/pCPP1232); 10, preparation made from *E.*

- 5 *coli*DH5α(pGP1-2/pBC SK (-)) in the same manner as 9. The picture was taken 3 days after infiltration.

Figure 5B shows suppression of the HrpW-induced HR by inhibitors of plant metabolism. Panels: 1, 5 mM KPO₄ buffer (pH 6.5); 2, HrpW CFEP; 3, HrpN CFEP + cycloheximide; 4, HrpW CFEP + LaCl₃; 5, HrpW CFEP + Na₃VO₄; 6, 10 HrpN CFEP; 7, HrpN CFEP + cycloheximide; 8, HrpN CFEP + Na₃VO₄; 9, PelE in 10 mM Tris-HCl (pH 7.8); 10, PelE + cycloheximide; 11, PelE + Na₃VO₄. CFEPs contain 0.1 mg/ml of HrpW or HrpN. The picture of the tobacco leaf was taken 36 hours after infiltration.

Figure 6 shows a Southern blot which indicates that *hrpW* of *E.* 15 *amylovora* Ea321 is present in other bacteria. Genomic DNA of strains was probed with a 1.4-kb *Hpa*I fragment that contains Ea321 *hrpW*. Lanes: 1, Ea321; 2, Ea266; 3, Ea273; 4, Ea246; 5, Ea510; 6, Ea528; 7, Ea574; 8, Ea546; 9, Ea557; 10, Ea562; 11, Ea587; 12, *E. carotovora* subsp. *carotovora* ATCC15713; 13, *E. mallotivora* 1818; 14, *E. salicis* 1822; 15, pCPP2157 of *E. chrysanthemi* EC16.

20

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an isolated DNA molecule having a nucleotide sequence of SEQ. ID. No. 1 as follows:

25

ATGTCAATTC TTACGCTTAA CAACAATACC TCGTCCTCGC CGGGTCTGTT CCAGTCCGGG 60

GGGGACAACG GGCTTGGTGG TCATAATGCA AATTCTGCGT TGGGGCAACA ACCCATCGAT 120

30

CGGCAAACCA TTGAGCAAAT GGCTCAATTA TTGGCGGAAC TGTTAAAGTC ACTGCTATCG 180

CCACAATCAG GTAATGCGGC AACCGGAGCC GGTGGCAATG ACCAGACTAC AGGAGTTGGT 240

35

AACGCTGGCG GCCTGAACGG ACGAAAAGGC ACAGCAGGAA CCACTCCGCA GTCTGACAGT 300

CAGAACATGC TGAGTGAGAT GGGCAACAAC GGGCTGGATC AGGCCATCAC GCCCGATGGC 360

CAGGGCGGCG GGCAGATCGG CGATAATCCT TTAAGTAAAG CCATGCTGAA GCTTATTGCA 420

40

CGCATGATGG ACGGCCAAAG CGATCAGTTT GGCCAACCTG GTACGGGCAA CAACAGTGCC 480

TCTTCCGGTA CTTCTTCATC TGGCGGTTCC CCTTTTAACG ATCTATCAGG GGGGAAGGCC 540
 5 CCTTCCGGCA ACTCCCCTTC CGGCAACTAC TCTCCCGTCA GTACCTTCTC ACCCCCATCC 600
 ACGCCAACGT CCCCTACCTC ACCGCTTGAT TTCCCTTCTT CTCCCACCAA AGCAGCCGGG 660
 GGCAGCACGC CGGTAACCGA TCATCCTGAC CCTGTTGGTA GCGCGGGCAT CGGGGCCGGA 720
 10 AATTCGGTGG CCTTCACCAG CGCCGGCGCT AATCAGACGG TGCTGCATGA CACCATTACC 780
 GTGAAAGCGG GTCAGGTGTT TGATGGCAAA GGACAAACCT TCACCGCCGG TTCAGAATTA 840
 GGCATGGCG GCCAGTCTGA AAACCAGAAA CCGCTGTTTA TACTGGAAGA CGGTGCCAGC 900
 15 CTGAAAAACG TCACCATGGG CGACGACGGG GCGGATGGTA TTCATCTTTA CGGTGATGCC 960
 AAAATAGACA ATCTGCACGT CACCAACGTG GGTGAGGACG CGATTACCGT TAAGCCAAAC 1020
 20 AGCGCGGGCA AAAAATCCCA CGTTGAAATC ACTAACAGTT CCTTCGAGCA CGCCTCTGAC 1080
 AAGATCCTGC AGCTGAATGC CGATACTAAC CTGAGCGTTG ACAACGTGAA GGCCAAAGAC 1140
 TTTGGTACTT TTGTACGCAC TAACGGCGGT CAACAGGGTA ACTGGGATCT GAATCTGAGC 1200
 25 CATATCAGCG CAGAAGACGG TAAGTTCTCG TTCGTTAAAA GCGATAGCGA GGGGCTAAAC 1260
 GTCAATACCA GTGATATCTC ACTGGGTGAT GTTGAAAACC ACTACAAAGT GCCGATGTCC 1320
 30 GCCAACCTGA AGGTGGCTGA ATGA 1344

See GenBank Accession No. U94513. The isolated DNA molecule of the present
 invention encodes a hypersensitive response elicitor protein or polypeptide having an
 35 amino acid sequence of SEQ. ID. No. 2 as follows:

Met Ser Ile Leu Thr Leu Asn Asn Asn Thr Ser Ser Ser Pro Gly Leu
 1 5 10 15
 40 Phe Gln Ser Gly Gly Asp Asn Gly Leu Gly Gly His Asn Ala Asn Ser
 20 25 30
 Ala Leu Gly Gln Gln Pro Ile Asp Arg Gln Thr Ile Glu Gln Met Ala
 35 40 45
 45 Gln Leu Leu Ala Glu Leu Leu Lys Ser Leu Leu Ser Pro Gln Ser Gly
 50 55 60
 50 Asn Ala Ala Thr Gly Ala Gly Gly Asn Asp Gln Thr Thr Gly Val Gly
 65 70 75 80
 Asn Ala Gly Gly Leu Asn Gly Arg Lys Gly Thr Ala Gly Thr Thr Pro
 85 90 95
 55 Gln Ser Asp Ser Gln Asn Met Leu Ser Glu Met Gly Asn Asn Gly Leu
 100 105 110


[illegible]

His Ile Ser Ala Glu Asp Gly Lys Phe Ser Phe Val Lys Ser Asp Ser
405 410 415

5 Glu Gly Leu Asn Val Asn Thr Ser Asp Ile Ser Leu Gly Asp Val Glu
420 425 430

Asn His Tyr Lys Val Pro Met Ser Ala Asn Leu Lys Val Ala Glu
435 440 445

10

 This protein or polypeptide is acidic, rich in glycine and serine, and lacks cysteine. It is also heat stable, protease sensitive, and suppressed by inhibitors of plant metabolism. The protein or polypeptide of the present invention has a predicted molecular size of ca. 4.5 kDa.

15

Fragments of the above hypersensitive response elicitor polypeptide or protein are encompassed by the the present invention.

20

Suitable fragments can be produced by several means. In the first, subclones of the gene encoding the elicitor protein of the present invention are produced by conventional molecular genetic manipulation by subcloning gene fragments. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller protein or peptide that can be tested for elicitor activity according to the procedure described below.

25

As an alternative, fragments of an elicitor protein can be produced by digestion of a full-length elicitor protein with proteolytic enzymes like chymotrypsin or *Staphylococcus* proteinase A, or trypsin. Different proteolytic enzymes are likely to cleave elicitor proteins at different sites based on the amino acid sequence of the elicitor protein. Some of the fragments that result from proteolysis may be active elicitors of resistance.

30

In another approach, based on knowledge of the primary structure of the protein, fragments of the elicitor protein gene may be synthesized by using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. These then would be cloned into an appropriate vector for increased expression of a truncated peptide or protein.

35

Chemical synthesis can also be used to make suitable fragments. Such a synthesis is carried out using known amino acid sequences for the elicitor being produced. Alternatively, subjecting a full length elicitor to high temperatures and

pressures will produce fragments. These fragments can then be separated by conventional procedures (e.g., chromatography, SDS-PAGE).

Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure and hydrophobic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

Suitable DNA molecules are those that hybridize to a DNA molecule comprising a nucleotide sequence of SEQ. ID. No. 1 under stringent conditions. An example of suitable stringency conditions is when hybridization is carried out at 65°C for 20 hours in a medium containing 1M NaCl, 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.1% sodium dodecyl sulfate, 0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 50 µg/ml *E. coli* DNA. However, any DNA molecules hybridizing to a DNA molecule comprising the nucleotide sequence of SEQ. ID. No. 1 under such stringent conditions must not be identical to the nucleic acids encoding the hypersensitive response elicitor proteins or polypeptides of *E. amylovora* (as disclosed by Wei, Z.-M., et al, "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992), which is hereby incorporated by reference), *Erwinia chrysanthemi* (as disclosed by Bauer, et. al., "*Erwinia chrysanthemi* Harpin_{Ech}: Soft-Rot Pathogenesis," MPMI 8(4): 484-91 (1995), which is hereby incorporated by reference), *Erwinia carotovora* (as disclosed by Cui, et. al., "The RsmA⁻ Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrpN*_{Ecc} and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI 9(7): 565-73 (1996), which is hereby incorporated by reference), *Erwinia stewartii* (as disclosed by Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microb. Inter. July 14-19, 1996 and Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," Ann. Mtg. Am. Phytopath. Soc. July 27-31, 1996), which are hereby incorporated by reference), and

Pseudomonas syringae pv. *syringae* (WO 94/26782 to Cornell Research Foundation, Inc., which is hereby incorporated by reference).

5 The protein or polypeptide of the present invention is preferably produced in purified form (preferably at least about 80%, more preferably 90%, pure) by conventional techniques. Typically, the protein or polypeptide of the present invention is secreted into the growth medium of recombinant host cells. Alternatively, the protein or polypeptide of the present invention is produced but not secreted into growth medium. In such cases, to isolate the protein, the host cell (e.g., *E. coli*) carrying a recombinant plasmid is propagated, lysed by sonication, heat, 10 differential pressure, or chemical treatment, and the homogenate is centrifuged to remove bacterial debris. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the polypeptide or protein of the present invention is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins. If necessary, the protein fraction 15 may be further purified by HPLC.

The DNA molecule encoding the hypersensitive response elicitor polypeptide or protein can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e. not normally 20 present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby 25 incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

30 Recombinant genes may also be introduced into viruses, such as vaccina virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see

5 "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F.W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced

10 into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference.

15 A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems

20 infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

25 Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promotor which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promotors differ from

30 those of procaryotic promotors. Furthermore, eucaryotic promotors and accompanying genetic signals may not be recognized in or may not function in a

procaryotic system, and, further, procaryotic promoters are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes.

5 Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably
10 promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

Promoters vary in their "strength" (i.e. their ability to promote
15 transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, *lac* promoter,
20 *trp* promoter, *recA* promoter, ribosomal RNA promoter, the P_R and P_L promoters of coliphage lambda and others, including but not limited, to *lacUV5*, *ompF*, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lacUV5 (tac)* promoter or other *E. coli* promoters produced by recombinant DNA or other synthetic DNA techniques may be
25 used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promoter unless specifically induced. In certain operations, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG
30 (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promotor, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the isolated DNA molecule encoding the hypersensitive response elicitor polypeptide or protein has been cloned into an expression system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like.

The present invention further relates to methods of imparting disease resistance to plants, enhancing plant growth, and/or effecting insect control for plants. These methods involve applying a hypersensitive response elicitor polypeptide or protein in a non-infectious form to all or part of a plant or a plant seed under conditions where the polypeptide or protein contacts all or part of the cells of the plant or plant seed. Alternatively, the hypersensitive response elicitor protein or polypeptide can be applied to plants such that seeds recovered from such plants themselves are able to impart disease resistance in plants, to enhance plant growth, and/or to effect insect control.

As an alternative to applying a hypersensitive response elicitor polypeptide or protein to plants or plant seeds in order to impart disease resistance in plants, to effect plant growth, and/or to control insects on the plants or plants grown from the seeds, transgenic plants or plant seeds can be utilized. When utilizing

transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein and growing the plant under conditions effective to permit that DNA molecule to impart disease resistance to plants, to enhance plant growth, and/or to control insects.

- 5 Alternatively, a transgenic plant seed transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein can be provided and planted in soil. A plant is then propagated from the planted seed under conditions effective to permit that DNA molecule to impart disease resistance to plants, to enhance plant growth, and/or to control insects.

- 10 The embodiment of the present invention where the hypersensitive response elicitor polypeptide or protein is applied to the plant or plant seed can be carried out in a number of ways, including: 1) application of an isolated elicitor polypeptide or protein; 2) application of bacteria which do not cause disease and are transformed with genes encoding a hypersensitive response elicitor polypeptide or protein; and 3) application of bacteria which cause disease in some plant species (but
15 not in those to which they are applied) and naturally contain a gene encoding the hypersensitive response elicitor polypeptide or protein.

- In one embodiment of the present invention, the hypersensitive response elicitor polypeptide or protein of the present invention can be isolated from
20 *Erwinia amylovora* as described in Examples *infra*. Preferably, however, the isolated hypersensitive response elicitor polypeptide or protein of the present invention is produced recombinantly and purified as described *supra*.

- In other embodiments of the present invention, the hypersensitive response elicitor polypeptide or protein of the present invention can be applied to
25 plants or plant seeds by applying bacteria containing genes encoding the hypersensitive response elicitor polypeptide or protein. Such bacteria must be capable of secreting or exporting the polypeptide or protein so that the elicitor can contact plant or plant seeds cells. In these embodiments, the hypersensitive response elicitor polypeptide or protein is produced by the bacteria *in planta* or on seeds or just prior to
30 introduction of the bacteria to the plants or plant seeds.

In one embodiment of the bacterial application mode of the present invention, the bacteria do not cause the disease and have been transformed (e.g.,

recombinantly) with genes encoding a hypersensitive response elicitor polypeptide or protein. For example, *E. coli*, which does not elicit a hypersensitive response in plants, can be transformed with genes encoding a hypersensitive response elicitor polypeptide or protein and then applied to plants. Bacterial species other than *E. coli* can also be used in this embodiment of the present invention.

In another embodiment of the bacterial application mode of the present invention, the bacteria do cause disease and naturally contain a gene encoding a hypersensitive response elicitor polypeptide or protein. Examples of such bacteria are noted above. However, in this embodiment, these bacteria are applied to plants or their seeds which are not susceptible to the disease carried by the bacteria. For example, *Erwinia amylovora* causes disease in apple or pear but not in tomato. However, such bacteria will elicit a hypersensitive response in tomato. Accordingly, in accordance with this embodiment of the present invention, *Erwinia amylovora* can be applied to tomato plants or seeds to impart disease resistance, enhance growth, or control insects without causing disease in that species.

The method of the present invention can be utilized to treat a wide variety of plants or their seeds to impart disease resistance, enhance growth, and/or control insects. Suitable plants include dicots and monocots. More particularly, useful crop plants can include: alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, turnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane. Examples of suitable ornamental plants are: *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

With regard to the use of the hypersensitive response elicitor protein or polypeptide of the present invention in imparting disease resistance, absolute immunity against infection may not be conferred, but the severity of the disease is reduced and symptom development is delayed. Lesion number, lesion size, and extent of sporulation of fungal pathogens are all decreased. This method of imparting disease resistance has the potential for treating previously untreatable diseases,

treating diseases systemically which might not be treated separately due to cost, and avoiding the use of infectious agents or environmentally harmful materials.

The method of imparting pathogen resistance to plants in accordance with the present invention is useful in imparting resistance to a wide variety of pathogens including viruses, bacteria, and fungi. Resistance, *inter alia*, to the following viruses can be achieved by the method of the present invention: *Tobacco mosaic virus* and *Tomato mosaic virus*. Resistance, *inter alia*, to the following bacteria can also be imparted to plants in accordance with present invention: *Pseudomonas solanacearum*, *Pseudomonas syringae* pv. *tabaci*, and *Xanthamonas campestris* pv. *pelargonii*. Plants can be made resistant, *inter alia*, to the following fungi by use of the method of the present invention: *Fusarium oxysporum* and *Phytophthora infestans*.

With regard to the use of the hypersensitive response elicitor protein or polypeptide of the present invention to enhance plant growth, various forms of plant growth enhancement or promotion can be achieved. This can occur as early as when plant growth begins from seeds or later in the life of a plant. For example, plant growth according to the present invention encompasses greater yield, increased quantity of seeds produced, increased percentage of seeds germinated, increased plant size, greater biomass, more and bigger fruit, earlier fruit coloration, and earlier fruit and plant maturation. As a result, the present invention provides significant economic benefit to growers. For example, early germination and early maturation permit crops to be grown in areas where short growing seasons would otherwise preclude their growth in that locale. Increased percentage of seed germination results in improved crop stands and more efficient seed use. Greater yield, increased size, and enhanced biomass production allow greater revenue generation from a given plot of land.

Another aspect of the present invention is directed to effecting any form of insect control for plants. For example, insect control according to the present invention encompasses preventing insects from contacting plants to which the hypersensitive response elicitor has been applied, preventing direct insect damage to plants by feeding injury, causing insects to depart from such plants, killing insects proximate to such plants, interfering with insect larval feeding on such plants, preventing insects from colonizing host plants, preventing colonizing insects from

releasing phytotoxins, etc. The present invention also prevents subsequent disease damage to plants resulting from insect infection.

The present invention is effective against a wide variety of insects. European corn borer is a major pest of corn (dent and sweet corn) but also feeds on
5 over 200 plant species including green, wax, and lima beans and edible soybeans, peppers, potato, and tomato plus many weed species. Additional insect larval feeding pests which damage a wide variety of vegetable crops include the following: beet armyworm, cabbage looper, corn ear worm, fall armyworm, diamondback moth, cabbage root maggot, onion maggot, seed corn maggot, pickleworm (melonworm),
10 pepper maggot, and tomato pinworm. Collectively, this group of insect pests represents the most economically important group of pests for vegetable production worldwide.

The method of the present invention involving application of the hypersensitive response elicitor polypeptide or protein can be carried out through a
15 variety of procedures when all or part of the plant is treated, including leaves, stems, roots, propagules (e.g., cuttings), etc. This may (but need not) involve infiltration of the hypersensitive response elicitor polypeptide or protein into the plant. Suitable application methods include high or low pressure spraying, injection, and leaf abrasion proximate to when elicitor application takes place. When treating plant
20 seeds, in accordance with the application embodiment of the present invention, the hypersensitive response elicitor protein or polypeptide can be applied by low or high pressure spraying, coating, immersion, or injection. Other suitable application procedures can be envisioned by those skilled in the art provided they are able to effect contact of the hypersensitive response elicitor polypeptide or protein with cells
25 of the plant or plant seed. Once treated with the hypersensitive response elicitor of the present invention, the seeds can be planted in natural or artificial soil and cultivated using conventional procedures to produce plants. After plants have been propagated from seeds treated in accordance with the present invention, the plants may be treated with one or more applications of the hypersensitive response elicitor
30 protein or polypeptide to impart disease resistance to plants, to enhance plant growth, and/or to control insects on the plants.

The hypersensitive response elicitor polypeptide or protein can be applied to plants or plant seeds in accordance with the present invention alone or in a mixture with other materials. Alternatively, the hypersensitive response elicitor polypeptide or protein can be applied separately to plants with other materials being applied at different times.

A composition suitable for treating plants or plant seeds in accordance with the application embodiment of the present invention contains a hypersensitive response elicitor polypeptide or protein in a carrier. Suitable carriers include water, aqueous solutions, slurries, or dry powders. In this embodiment, the composition contains greater than 500 nM hypersensitive response elicitor polypeptide or protein.

Although not required, this composition may contain additional additives including fertilizer, insecticide, fungicide, nematocide, and mixtures thereof. Suitable fertilizers include $(\text{NH}_4)_2\text{NO}_3$. An example of a suitable insecticide is Malathion. Useful fungicides include Captan.

Other suitable additives include buffering agents, wetting agents, coating agents, and abrading agents. These materials can be used to facilitate the process of the present invention. In addition, the hypersensitive response elicitor polypeptide or protein can be applied to plant seeds with other conventional seed formulation and treatment materials, including clays and polysaccharides.

In the alternative embodiment of the present invention involving the use of transgenic plants and transgenic seeds, a hypersensitive response elicitor polypeptide or protein need not be applied topically to the plants or seeds. Instead, transgenic plants transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein are produced according to procedures well known in the art.

The vector described above can be microinjected directly into plant cells by use of micropipettes to transfer mechanically the recombinant DNA. Crossway, Mol. Gen. Genetics, 202:179-85 (1985), which is hereby incorporated by reference. The genetic material may also be transferred into the plant cell using polyethylene glycol. Krens, et al., Nature, 296:72-74 (1982), which is hereby incorporated by reference.

Another approach to transforming plant cells with a gene which imparts resistance to pathogens is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the heterologous DNA. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the vector and heterologous DNA) can also be propelled into plant cells.

Yet another method of introduction is fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible lipid-surfaced bodies. Fraley, et al., Proc. Natl. Acad. Sci. USA, 79:1859-63 (1982), which is hereby incorporated by reference.

The DNA molecule may also be introduced into the plant cells by electroporation. Fromm et al., Proc. Natl. Acad. Sci. USA, 82:5824 (1985), which is hereby incorporated by reference. In this technique, plant protoplasts are electroporated in the presence of plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

Another method of introducing the DNA molecule into plant cells is to infect a plant cell with *Agrobacterium tumefaciens* or *A. rhizogenes* previously transformed with the gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Agrobacterium is a representative genus of the gram-negative family Rhizobiaceae. Its species are responsible for crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes*). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized only by the bacteria. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In addition, assaying for the presence of opines can be used to identify transformed tissue.

Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of *A. tumefaciens* or the Ri plasmid of *A. rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells on infection by Agrobacterium and is stably integrated into the plant genome. J. Schell, Science, 237:1176-83 (1987), which is hereby incorporated by reference.

After transformation, the transformed plant cells must be regenerated.

Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co., New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. III (1986), which are hereby incorporated by reference.

It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of sugarcane, sugar beets, cotton, fruit trees, and legumes.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable.

After the expression cassette is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Once transgenic plants of this type are produced, the plants themselves
5 can be cultivated in accordance with conventional procedure with the presence of the gene encoding the hypersensitive response elicitor resulting in disease resistance, enhanced plant growth, and/or control of insects on the plant. Alternatively, transgenic seeds are recovered from the transgenic plants. These seeds can then be planted in the soil and cultivated using conventional procedures to produce transgenic
10 plants. The transgenic plants are propagated from the planted transgenic seeds under conditions effective to impart disease resistance to plants, to enhance plant growth, and/or to control insects. While not wishing to be bound by theory, such disease resistance, growth enhancement, and/or insect control may be RNA mediated or may result from expression of the elicitor polypeptide or protein.

15 When transgenic plants and plant seeds are used in accordance with the present invention, they additionally can be treated with the same materials as are used to treat the plants and seeds to which a hypersensitive response elicitor polypeptide or protein is applied. These other materials, including hypersensitive response elicitors, can be applied to the transgenic plants and plant seeds by the above-noted procedures,
20 including high or low pressure spraying, injection, coating, and immersion. Similarly, after plants have been propagated from the transgenic plant seeds, the plants may be treated with one or more applications of the hypersensitive response elicitor to impart disease resistance, enhance growth, and/or control insects. Such plants may also be treated with conventional plant treatment agents (e.g., insecticides, fertilizers, etc.).

25

EXAMPLES

Example 1 - Bacterial Strains and Plasmids

30 *E. amylovora* Ea321 and Ea273 are wild-type strains that infect pomaceous plants (Beer et al., The hrp Gene Cluster of *Erwinia amylovora*, eds. Hennecke, H. & Verma, D.P.S. (Kluwer Academic Publishers, Dordrecht, The Netherlands), Vol. 1, pp. 53-50 (1991), which is hereby incorporated by reference).

Escherichia coli DH5 α was used routinely as the host of plasmids. pCPP1012 is a subclone of pCPP430, and pCPP1152, pCPP1218, pCPP1219 and pCPP1220 were constructed by cloning restriction fragments of pCPP1012 into pBluescript KS (+) (Stratagene, La Jolla, CA) (Fig. 1B). pCPP1227 was cloned from pCPP 1220 into the same vector.

Example 2 - Molecular Biological Techniques and Sequence Analysis.

General molecular procedures were performed using standard techniques as described (Sambrook, et al., Molecular Cloning: A Laboratory Manual, (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (1989), which is hereby incorporated by reference). Sequencing was done on an ABI 373A automated DNA sequencer at the Cornell University Biotechnology Program DNA Sequencing Facility. For DNA and protein sequence analyses, programs in the GCG software package, Version 7.3 (Genetics Computer Group, Inc., Madison, WI) and DNASTAR (DNASTAR, Inc., Madison, WI) were used.

Example 3 - Expression of *hrpW* in *E. coli*.

The 1.4-kb *Hpa*I fragment of pCPP1227 that contains *hrpW* was subcloned into pBC SK (-) (Stratagene, La Jolla, CA) such that *hrpW* is under the control of T7 Φ 10 promoter. The resulting plasmid, pCPP1232 (Fig. 1B), was introduced into *E. coli* DH5 α (pGP1-2) (Tabor, et al., Proc. Natl. Acad. Sci. USA, 82:1074-78 (1985), which is hereby incorporated by reference). Cells were incubated at 42°C to induce the expression of the T7 RNA polymerase gene, and newly synthesized proteins were radiolabelled with ³⁵S-Met as described (Tabor, et al., Proc. Natl. Acad. Sci. USA, 82:1074-78 (1985), which is hereby incorporated by reference). Resulting samples were resuspended in a crackling buffer and heated to 95°C for 3 min before SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% gel.

Example 4 - Purification of HrpW.

HrpW, produced by heat-shock treatment of *E. coli* DH5 α (pGP1-2, pCPP1232) at 42°C, was purified by cutting out the area of the gel containing HrpW, eluting the protein with ELUTRAP (Schleicher & Schuell, Inc., Keene, NH), and

desalting the HrpW-containing solution using Centriprep-30 (Amicon, Inc., Beverly, MA) and 5mM potassium phosphate (KPO₄) buffer (pH 6.5). Alternatively, heat-induced and 10-fold concentrated *E. coli* DH5 α (pGP1-2, pCPP1232) cells were sonicated in the presence of 1mM phenylmethanesulfonyl fluoride (PMSF), put in a boiling water bath for 10 min, and centrifuged at 17,500 g for 10 min. The supernatant was desalted resulting in a "cell-free elicitor preparation (CFEP)" of HrpW. The CFEP of HrpW was prepared in the same manner from an HrpN overproducer, *E. coli* DH5 α (pCPP2139).

10 **Example 5 - Immunodetection of HrpW.**

Polyclonal antibodies against HrpW were raised at the College of Veterinary Medicine, Cornell University, by injecting ca. 100 μ g of HrpW into a rabbit three times at 2-3 wk intervals. The antiserum was collected 2 wk after the final injection and cross-absorbed with heat-treated lysate of *E. coli* DH5 α (pGP1-2, pBC SK (-)).

15 *E. amylovora* Ea321Rp (a rifampicin-resistant derivative of Ea321), Ea321-K49 (*hrpL*::Tn 10-miniKm) (Wei, et al., J. Bacteriol., 177:6201-10 (1995), which is hereby incorporated by reference), Ea321-G84 (*hrcC*::Tn5-*gusA1*) (Kim et al., J. Bacteriol., 179:1690-97 (1997), which is hereby incorporated by reference),
20 Ea273Rp, Ea273-K49, and Ea273-G73 (*hrcV*::Tn5-*gusA1*) were grown overnight in Terrific broth, transferred to a *hrp* minimal medium (Huynh, et al., Science, 345:1374-77 (1989), which is hereby incorporated by reference) at 1H10⁸ cfu/ml, and incubated at 20°C until the bacteria grew to 1H10⁹ cfu/ml. Cultures were centrifuged at 17,500 g and the pellet was resuspended in a loading buffer. The supernatant was
25 passed through a membrane filter (0.2 μ m pore size; Whatman Inc., Fairfield, NJ) after adding 1mM PMSF, and concentrated 100-fold using Centricon-10 and Microcon-10 (Amicon, Inc., Beverly, MA) at 4°C. Both the cell and supernatant fractions were then subjected to SDS-PAGE in a 10% gel.

Proteins in the gel were transferred to Immobilon-P (Millipore Co., Bedford, MA) and western analysis was performed using a system (Sigma, St. Louis, MO) composed of Biotin-conjugated anti-rabbit IgG, ExtrAvidin, and BCIP/NBT

tablets for strains of Ea273 and *E. coli*, and using the Western-Light Plus system (Tropix, Inc., Bedford, MA) for strains of Ea321.

Example 6 - Generation of an N-terminal Fragment of HrpW.

5 pCPP1232 was digested with *Bam*HI and *Bst*EII and the ends of the 4.1-kb fragment were blunted using the Klenow fragment and self-ligated. The resulting plasmid, pCPP1254, which encodes the N-terminal 226 amino acids of HrpW and Ile-His residues derived from the vector sequence, was cloned in *E. coli* DH5 α , and then transferred to *E. coli* DH5 α (pGP1-2), generating *E. coli* 10 DH5 α (pGP1-2, pCPP1254).

Example 7 - Plant Assays.

52b
D3 7
15 Elicitation of the HR was tested by infiltrating protein or bacterial preparations into the intercellular space of leaves of tobacco (*Nicotiana tabacum* L. 'xanthi') and other plants (Kim, et al., J. Bacteriol., 179:1690-97 (1997), which is hereby incorporated by reference). Cells were grown either in Luria broth (*E. coli* DH5 α and MC4100) or a *hrp* minimal medium (*E. amylovora* Ea321 and Ea321-T5) (Huynh, et al., Science, 345:1374-77 (1989), which is hereby incorporated by reference) to 5H10⁸ cfu/ml, and resuspended in 5 mM KPO₄ buffer (pH 6.5) to 2H10⁸ 20 cfu/ml (*E. coli* strains) or 5H10⁸ cfu/ml (*E. amylovora* strains). Inhibitors of plant metabolism used included cycloheximide at 100 μ M, LaCl₃ at 1 mM, and Na₃VO₄ at 50 μ M.

Example 8 - Southern Blotting.

52b
D4
25 7 Genomic DNA was digested with *Eco*RI, electrophoresed on a 0.7% agarose gel, transferred to an Immobilon-N membrane (Millipore Co., Bedford, MA), and hybridized with the ³²P-Labelled 1.4kb-*Hpa*I fragment of pCPP1227 at 65°C for 24 hr. The membrane was washed twice with a solution of 2H SCC and 1.0% SDS at 65°C, and washed with 0.1H SCC until no radioactivity is detected in the wash 30 solution. For low stringency hybridizations, the membrane was incubated at 50°C and washed with 2H SCC at 45°C.

Example 9 - Pectic Enzyme Assay of HrpW.

Heat-induced *E. coli* DH5 α (pGP1-2, pCPP1232) were pelleted, resuspended in one-tenth volume of 5 mM KPO₄ buffer (pH 6.5) or 10 mM Tris-HCl (pH 8.5), sonicated on ice, centrifuged, and PL activity of the supernatant was tested. Also, 50-fold concentrated Ea321 culture supernatant was included in the test. Dilute PelE of *Erwinia Chrysanthemi* EC16 in 10 mM Tris-HCl (pH 7.8) was used as a control.

Ten microliters of each preparation was spotted in YC agar plates (Keen, et al., J. Bacteriol., 159:825-31 (1984), which is hereby incorporated by reference) containing either 0.7% polygalaturonic acid (Sigma, St. Louis, MO) or 0.7% pectin (88% methoxylated; Sigma, St. Louis, MO) at pH 6.5, 8.0 or 9.5, and in pectin semi-solid agar plates (Starr, et al., J. Clin. Microbiol., 6:379-86 (1977), which is hereby incorporated by reference) containing 3% pectin (88% methoxylated) at pH 6.5, 8.0 or 9.5. The plates were incubated at 37°C for 24 hr, flooded with 1 M CaCl₂, and examined for the presence of halo. Viscometric analysis (Bateman, D.F., Phytopathology, 53:197-204 (1963), which is hereby incorporated by reference) and a modified thioarbituric acid procedure (Sherwood, R.T., Phytopathology, 56:279-86 (1966), which is hereby incorporated by reference) was done using 1% polygalaturonic acid or 1% pectin (68% methoxylated) in 2.5 mM CaCl₂ and 100 mM Tris-HCl, pH 9.5 as substrates. Methods supposedly more sensitive than those above, including an isoelectrofocusing gel procedure and spectrophotometry also were tried. Ten microliters of samples was applied onto an overlay gel (Collmer, et al., J. Bacteriol., 161:913-20 (1985), which is hereby incorporated by reference) containing 0.2% pectin (88% methoxylated), 1% agarose, 1.5 mM CaCl₂, and 50 mM Tris-HCl (pH 8.8), and wrapped with plastic film. The gel was incubated at 28°C for 24 hr, flooded with 1% hexadecyltrimethylammonium bromide, and inspected for clearing. To assay PL activity through generation of double bonds, 1.9 ml of a solution of 0.1% pectin (68% methoxylated), 5 mM CaCl₂, and 100 mM Tris-HCl at pH 5.5 or 9.5 was mixed with 100 μ l of samples and absorbance changes at 232 nm was recorded for 30 min as described (Alfano, et al., J. Bacteriol., 177:4553-56 (1995), which is hereby incorporated by reference).

Example 10 - Identification of a Gene Encoding a Glycine-Rich Protein.

hrpN mutants pCPP430-T5 and pCPP450-T5 in *E. coli* exhibited residual HR-eliciting activity (Fig. 5A, panels 2 and 4), suggesting the existence of another HR elicitor in the clones. The DNA downstream of *hrpN*, where pCPP430 and pCPP450 overlap, therefore, was subcloned and its sequence was determined. This revealed four open reading frames, designated ORF A, ORF B, ORF C and *hrpW* (Fig. 1B). A putative HrpL-dependent promoter (Bogdanove, et al., *J. Bacteriol.*, 178:1720-30 (1996) and Kim et al., *J. Bacteriol.*, 179:1690-97 (1997), which are hereby incorporated by reference), CGGAACC-N₄-C-N₁₀-CCACTCAAT (SEQ. ID. No. 3), was found 58-bp upstream of the *hrpW* start codon, suggesting that the expression of *hrpW* is controlled by HrpL, an alternate sigma factor (Wei, et al., *J. Bacteriol.*, 177:6201-10 (1995), which is hereby incorporated by reference). *hrpW* in pCPP1232 (Fig. 1B) was expressed using a T7 RNA polymerase/promoter system, and a specific protein band with an apparent molecular weight of ca. 60-kDa resulted (Fig. 2). This is larger than its expected size of 45-kDa. The same size band, however, was observed from the supernatant of *E. amylovora* (Fig. 4), indicating that the aberrant size of the protein is not a cloning artifact.

Example 11 - Predicted Features of the *hrpW* Product.

hrpW was deduced to encode a protein of 447-aa residues, which is acidic (pI-4.5), hydrophilic, rich in Gly, Ser, and Asn, low in Glu, Arg, Trp, and Tyr, and lacking in Cys (Fig. 3). These properties are similar to harpins, although the primary structure of HrpW seemed not homologous to any of them. The sequence of HrpW suggests that the protein is composed of two domains: the N-terminal Gly- and Ser-rich domain and the C-terminal domain homologous to PLs (see below). About two-thirds of Gly and Ser are located in the N-terminal region. The Gly and Ser content of the first 240-aa residues is 17.5% and 14.2%, respectively. The N-terminal region could be divided into five subregions, and contained two sequences (residues 40-59 and 131-145) that may form amphipathic α -helices. The first 39 residues of the N-terminus contains many Gly, Ser, Leu, and Asn, but few charged or aromatic amino acids. Similarly, the region that connects the two potential α -helices has high Gly,

Asn, and Gln content, but no aromatic residues. Residues 146-232 contain several repeats of Ser/Thr-Pro/Ser/Thr-Pro/Ser/Thr, suggesting that this region might be a linker (Gilkes, et al., Microbiol. Rev., 55:303-15 (1991), which is hereby incorporated by reference).

5

Example 12 - C-terminus of HrpW is Homologous to Pectate Lyases.

Database searches using BLAST and FASTA algorithms (Altschul, et al., J. Mol. Biol., 215:403-10 (1990) and Pearson, et al., Proc. Natl. Acad. Sci. USA, 85:2444-48 (1988), which are hereby incorporated by reference) indicated that the

10 C-terminal region of HrpW is homologous to PLA-D of *Nectria haematococca* mating type VI (*Fusarium solani* f. sp. *pisi*) (Gonzalez, et al., J. Bacteriol., 174:6343-49 (1992), Guo, et al., J. Bacteriol., 177: 7070-77 (1995), Guo, et al., Arch. Biochem. Biophys., 323:352-60 (1995), and Guo, et al., Arch. Biochem. Biophys., 332:305-12 (1996), which are hereby incorporated by reference). BLAST P() values and FASTA

15 E() values from runs with default parameters were 4.0e-14 to 3.03-10 and 2.7e-08 to 1e-06, respectively. Based on BESTFIT alignments, HrpW was 27-33% identical to the fungal PLs and the Z-scores were 8.14 to 13.3 Also, database search with PLs of *N. haematococca* showed that they are homologs of Pel-3 and PelB of *Erwinia carotovora* subsp. *carotovora* (Liu, et al., Appl. Env. Microbiol., 60:2545-52 (1994)

20 and Heikinheimo, et al., Mol. Plant-Microbe Interact., 8:207-17 (1995), which are hereby incorporated by reference) (BLASTP P() values ranged from 9.0e-15 to 8.6e-10, and BESTFIT identities were 31-36%). These fungal PLs and *E. carotovora* Pel-3/PelB, together with HrpW, form a class distinct from other PL families. From an alignment of the proteins, five highly conserved blocks were recognizable (Fig. 3).

25 The seven members share 20 identical residues of which five are Gly. The PHD algorithm predicted β -sheets and loops for the PL-homology region of HrpW, except for the sequence at residues 329-336 which has a propensity to form an α -helix (Fig. 3). Intriguingly, HrpW does not contain any Cys, which are conserved among PLs in the class. In addition, PL activity of HrpW was not detected using the several tests

30 described in the materials and methods.

Example 13 - Production and Secretion of HrpW are *hrp*-dependent.

An immunoblot with anti-HrpW antibodies detected HrpW only from supernatant preparations of *E. amylovora* Ea321 and Ea273, indicating that HrpW is efficiently secreted (Fig. 4). HrpW was not found in preparations from *hrpL* mutants Ea321-K49 and Ea273-K49, demonstrating that expression of *hrpW* is *hrpL*-dependent. In addition, HrpW either was not detected or restricted to the whole cell preparations of *hrp* secretion mutants Ea321-G84 and Ea273-G73, respectively. Thus, secretion of HrpW is Hrp pathway-dependent. Anti-HrpW antibodies did not react with HrpW (Fig. 4, lane 2), suggesting structural differences between the two elicitors.

Example 14 - HrpW Induces Rapid Tissue Necrosis on Plants in a Heat-stable and Protease-sensitive Manner.

From the predicted properties of HrpW, it is inferred to be an HR elicitor. To test this possibility, the partially purified protein was infiltrated into tobacco leaves. The infiltrated area began to collapse after 8-12 hr, and typical tissue necrosis, indistinguishable from that elicited by HrpN, developed 24-36 hr after inoculation (Fig. 5A, panel 9). HrpW induced tissue necrosis in tobacco at concentrations of 1.1 μ M (50 μ g/ml). HrpW also caused necrosis in African violet, geranium, tomato, pepper, *Kalanchoe diargremontiana*, and *Arabidopsis thaliana*, but not in soybean. A heat-treated preparation of HrpW still caused rapid necrosis in tobacco leaves, indicating the heat-stable nature of the activity (Fig. 5A, panel 2). On the other hand, treatment of HrpW with 3 mg/ml protease (type XIV; Sigma, St. Louis, MO) for 1 hr destroyed HR-eliciting activity.

Example 15 - Elicitation by HrpW Requires Plant Metabolism.

A major question was whether the tissue necrosis caused by HrpW is due to a mechanism comparable to harpins (He, et al., Cell, 73:1255-66 (1993) and He, et al., Mol. Plant-Microbe Interact., 7:289-92 (1994), which are hereby incorporated by reference). Coinfiltration of HrpW CFEP with the metabolic inhibitors cycloheximide, lanthanum chloride, or sodium vanadate (targets are 80S ribosome, Ca²⁺ channels, ATPases/Y-phosphatases, respectively) prevented the HR

(Fig. 5B, panels 3-5), like HrpN CFEP with the inhibitors (Fig. 5B, panels 7-8). This indicates that active plant metabolism is needed for the HrpW-induced HR. Tobacco leaves infiltrated with PelE of *E. chrysanthemi* EC16 also exhibited rapid tissue necrosis (Fig. 5B, panel 9). However, necrosis caused by PelE occurred faster and the collapsed area was translucent, darker, softer, and easily crushed as compared to that elicited by harpins. In addition, PelE induced tissue necrosis irrespective of the presence of inhibitors (Fig. 5B, panels 10-11).

Example 16 - N-terminal Region is Sufficient for HR Elicitation.

A fragment of *hrpW* encoding the N-terminal 226 residues, designated HrpW(1-226), was constructed, and the production of HrpW(1-226) was confirmed. Typical HR developed 24-36 hr after infiltration of HrpW(1-226) CFEP into tobacco leaves, though the activity was weaker than that of full-length HrpW. That HrpW(1-226) are produced stably and elicits the HR independently of the C-terminal region support the two-domain structure of HrpW derived from the sequence data.

Example 17 - *hrpW* is Conserved Among Strains of *E. amylovora*.

The presence of *hrpW* in other bacteria was examined by Southern hybridization. Under high stringency conditions, single bands were observed for each of the ten strains of *E. amylovora* tested. The sizes of the restriction bands suggested three different groups. When low stringency conditions were used, hybridizing bands were visible from several other species of *Erwinia* such as *E. carotovora*, and *E. salicis*, and pCPP2157, a clone containing the *hrp* gene cluster of *E. chrysanthemi* (Bauer, et al., Mol. Plant-Microbe Interact., 8:484-91 (1995), which is hereby incorporated by reference) (Fig. 6).

Previously, at most only one harpin was known from each bacterium. However, *E. amylovora* encodes HrpW, a harpin distinct from the first harpin described (HrpN; Wei et al., Science, 257:85-88 (1992), which is hereby incorporated by reference). Southern analysis suggests that *hrpW* exists in several other *Erwinia* species, suggesting a role for HrpW in pathogenesis. Furthermore, sequence comparison indicated that EXP-60 (renamed to HrpW of *P. syringae* pv. tomato

(Yuan, et al., J. Bacteriol., 178:6399-6402 (1996), which is hereby incorporated by reference) is homologous to HrpW of *E. amylovora*.

Analysis suggests that HrpW is a multidomain protein comprised of the N-terminal Gly/Ser-rich domain and the C-terminal PL-homology domain. That HrpW has homology to PLs was surprising, because *E. amylovora* is believed to be non-pectolytic (Seemüller, et al., Phytopathology, 66:433-36 (1976), which is hereby incorporated by reference). Besides, no pectic enzyme function has been suggested for harpins (Alfano, et al., Plant Cell, 8:1683-98 (1996), which is hereby incorporated by reference). Although PL activity was not detected, HrpW is not the first PL homolog for which pectic enzyme activity function has not been demonstrated. Some plant pollen proteins are PL homologs and pectic enzyme function has been suspected from the pollen-specific expression of the encoding genes (Wing, et al., Plant Mol. Biol., 14:17-28 (1990), which is hereby incorporated by reference); however, they do not have detectable enzyme activity (Dircks, et al., Plant Physiol. Biochem., 34:509-20 (1996), which is hereby incorporated by reference). HrpW may differ in substrate specificity from its homologs, or it may lack the pectic enzyme function like α -lactalbumins, which are homologs of lysozymes but do not have lysozyme function (McKenzie, et al., Adv. Prot. Chem., 41:173-315 (1991), which is hereby incorporated by reference). Alternatively, instead of lyase function, HrpW may have only a pectic substance-binding function (Kurosky, et al., Proc. Natl. Acad. Sci. USA, 77:3388-92 (1980), which is hereby incorporated by reference).

HrpW is exceptional as a PL homolog in several respects. It does not possess the N-terminal signal peptide that is recognized by the Sec machinery; rather, it is secreted via a type III pathway. It does not contain any Cys residues conserved in PLs, which may have structural and functional roles. Furthermore, HR elicitation by HrpW depends on plant metabolism. Southern data suggests that *E. caratovora* has a *hrpW* homolog different from *pel-3/pelB*. Considering its dependence on the *hrp* system and the lack of detectable PL activity, the role of HrpW in pathogenesis may not be for simple degradation of cell wall for utilization as a carbon source; rather, through possible pectic enzyme activity or cell wall-binding activity, it may assist the Hrp pilus (Roine, et al., Proc. Natl. Acad. Sci. USA, 94:3459-64 (1997), which is

hereby incorporated by reference) or other pathogenicity/virulence/avirulence proteins in getting through the cell wall.

Several evolutionary related groups of pectic enzymes are apparent based on amino acid comparisons and structural analyses: i) a class called the
5 "extracellular PL superfamily" (Henrissat, et al., Plant Physiol., 107:963-76 (1995), and references therein, which are hereby incorporated by reference) that includes two PL families of plant pathogenic bacteria, *Bacillus subtilis* and some fungi, PelX of *E. carotovora*, pectin lyases, and plant pollen and style proteins, ii) a class comprising periplasmic PLs of *Yersinia pseudotuberculosis* and *E. carotovora* (Hinton, et al.,
10 Mol. Microbiol., 3:1785-96 (1989), which is hereby incorporated by reference) and KdgC of *E. chrysanthemi* (Condemine, et al., Mol. Microbiol., 5:2191-2202 (1991), which is hereby incorporated by reference), iii) a class that consists of PLs of *N. haematococca*, and Pel-3/PelB of *E. carotovora*, iv) PelX and PelL of
15 *E. chrysanthemi* (Alfano, et al., J. Bacteriol., 177:4553-56 (1995) and Lojkowska, et al., Mol. Microbiol., 16:1183-95 (1995), which are hereby incorporated by reference), and v) recently reported PelZ of *E. chrysanthemi* and *E. carotovora* (Pissavin, et al., J. Bacteriol., 178:7187-96 (1996), which is hereby incorporated by reference). The third class of homology group has not yet been recognized as a protein family, although members have been mentioned in the literature (Henrissat, et al., Plant Physiol.,
20 107:963-76 (1995) and Liao, et al., Mol. Plant-Microbe Interact., 9:14-21 (1996), which are hereby incorporated by reference). Therefore, this third class, to which HrpW of *E. amylovora* belongs, will be referred to as "class III pectate lyases" to differentiate it from the two earlier classes, which should be called "class I" and "class II". Members of the "class III PLs" appear to be widespread among plant
25 pathogens. Besides HrpW in *P. syringae* pv. tomato, *E. chrysanthemi* has Pell which is very closely related to Pel-3/PelB of *E. carotovora*.

It is enigmatic how harpins apparently heterogeneous in sequence and possibly in structure can induce the same plant response. The "cell-killing" action of harpins appears not due to potential enzymatic or toxic function such as making pores
30 in the cell membrane; HR activity is heat-stable (Wei, et al., Science, 257:85-88 (1992), He, et al., Cell, 73:1255-66 (1993), and Arlat, et al., EMBO J., 13:543-53 (1994), which are hereby incorporated by reference), requires plant metabolism (He,

et al., Cell, 73:1255-66 (1993), and He, et al., Mol. Plant-Microbe Interact., 7:289-92 (1994), which are hereby incorporated by reference), and fragments can elicit the reaction (Arlat, et al., EMBO J., 13:543-53 (1994), Alfano, et al., Mol. Microbial., 19:715-28 (1996), and Laby, et al., Molecular Studies on Interactions Between
5 *Erwinia amylovora* and Its Host and Non-Host Plants, Cornell University, Ithaca, NY (1997), which are hereby incorporated by reference). Avr proteins induce the HR on plants carrying corresponding resistance genes (Staskawicz, et al., Science, 268:661-67 (1995), which is hereby incorporated by reference). It seems less probable that harpins evoke the HR by the same kind of mechanism, although downstream
10 signaling events could be shared. Possible signaling mechanisms that lead to the HR against harpins were discussed by Novacky and colleagues (Hoyos, et al., Mol. Plant-Microbe Interact., 9:608-16 (1996), which is hereby incorporated by reference).

It appears that Gly/Ser richness may be important for HR-eliciting function of harpins, because non-overlapping fragments that elicit the HR include
15 Gly/Ser-rich regions (Arlat, et al., EMBO J., 13:543-53 (1994), Alfano, et al., Mol. Microbial., 19:715-28 (1996), and Laby, et al., Molecular Studies on Interactions Between *Erwinia amylovora* and Its Host and Non-Host Plants, Cornell University, Ithaca, NY (1997), which are hereby incorporated by reference). In support of the hypothesis, a truncated HrpW containing the N-terminal Gly/Ser-rich domain has HR-
20 eliciting ability. On the other hand, HR elicitation by fragments is weaker as compared to whole protein (Laby, et al., Molecular Studies on Interactions Between *Erwinia amylovora* and Its Host and Non-Host Plants, Cornell University, Ithaca, NY (1997) and this work, which are hereby incorporated by reference) indicating that
25 other part(s) of harpins contribute to the full-strength HR. It will be of interest to determine whether plant cell wall Gly-rich proteins ("GRPs"), the encoding genes of which are expressed during xylogenesis and after wounding or viral infection (Showalter, A.M., Plant Cell, 5:9-23 (1993), which is hereby incorporated by reference), possess the ability to cause cell death.

Harpins appear to be targeted to outer parts of plant cells such as the
30 cell wall. They can elicit the HR when exogenously applied to plant tissue by infiltration. When harpins are added to cell-suspension culture, K⁺ efflux and alkalinization of the medium, referred to as exchange reaction ("XR"), followed by

cell death occurs (Wei, et al., Science, 257:85-88 (1992) and Popham, et al., Physiol. Mol. Plant Pathol., 47:39-50 (1995), which is hereby incorporated by reference).

However, the XR does not occur in protoplast culture. In addition, HrpZ antibodies localize HrpZ outside of plant cells and not in protoplasts, and the alkalization and the localization is blocked by a chelating agent that extracts Ca^{2+} and pectin (Hoyos, et al., Mol. Plant-Microbe Interact., 9:608-16 (1996), which is hereby incorporated by reference). The homology of HrpW to PLs is consistent with a model in which the site of harpin action is the plant cell wall.

Type III systems of animal pathogens secrete many proteins involved in pathogenesis (for example, see Cornelis, et al., Mol. Microbiol., 23:861-67 (1997), which is hereby incorporated by reference). Until recently, however, only harpins have been shown to be delivered by the type III machinery of plant pathogens. Recent evidence suggests that multiple proteins are secreted through the Hrp pathway, and that several Avr proteins are transferred directly into the plant cell by way of the Hrp secretion machinery (Gopalan, et al., Plant Cell, 8:1095-1105 (1996), Leister, et al., Proc. Natl. Acad. Sci. USA, 93:15497-15502(1996), Scofield, et al., Science, 274:2063-65 (1996), Tang, et al., Science, 274:2060-63 (1996), and Van Den Ackerveken, et al., Cell, 87:1307-16 (1996), which are hereby incorporated by reference).

It is interesting that *hrpW* is flanked by *dspE* and ORF B (Fig. 1B), which are homologs of *avrE* of *P. syringae* and *avrRxv* of *X. campestris* pv. vesicatoria, respectively. The linkage of harpin genes and homologs of non-host *avr* genes provides a hint of relationships between them in pathogenesis. Harpins might in reality be a class of Avr proteins, or Avr proteins may be actually virulence proteins. PopA of *P. solanacearum* GMI1000 elicits the HR only in resistant petunia lines (Arlat, et al., EMBO J., 13:543-53 (1994), which is hereby incorporated by reference). Also, expression of the Avr phenotype is controlled by the *hrp* system, and some *avr* genes possess virulence or pathogenicity functions (Dangl, Curr. Top. Microbiol. Immunol., 192:99-118 (1994), which is hereby incorporated by reference). Indeed, *dspE* is a pathogenicity factor. Thus, the region of the *E. amylovora* genome where harpin genes and *avr* homologs reside may constitute an arsenal for proteins used to bombard different parts of the host cell. Elucidating their specific targets and

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations
5 can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Kim, Jihyun Francis
Beer, Steven V.
- (ii) TITLE OF INVENTION: HYPERSENSITIVE RESPONSE ELICITOR FROM
ERWINIA AMYLOVORA AND ITS USE
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Nixon, Hargrave, Devans & Doyle LLP
 - (B) STREET: P.O. Box 1051, Clinton Square.
 - (C) CITY: Rochester
 - (D) STATE: New York
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 14603
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 60/055,108
 - (B) FILING DATE: 06-AUG-1977
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Goldman, Michael L.
 - (B) REGISTRATION NUMBER: 30,727
 - (C) REFERENCE/DOCKET NUMBER: 19603/1581
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (716) 263-1304
 - (B) TELEFAX: (716) 263-1600

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1344 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

| | |
|--|------|
| ATGTCAATTC TTACGCTTAA CAACAATACC TCGTCCTCGC CGGGTCTGTT CCAGTCCGGG | 60 |
| GGGGACAACG GGCTTGGTGG TCATAATGCA AATTCTGCGT TGGGGCAACA ACCCATCGAT | 120 |
| CGGCAAACCA TTGACCAAAT GGCTCAATTA TTGGCGGAAC TGTTAAAGTC ACTGCTATCG | 180 |
| CCACAATCAG GTAATGCGGC AACCGGAGCC GGTGGCAATG ACCAGACTAC AGGAGTTGGT | 240 |
| AACGCTGGCG GCCTGAACGG ACGAAAAGGC ACAGCAGGAA CCACTCCGCA GTCTGACAGT | 300 |
| CAGAACATGC TGAGTGAGAT GGGCAACAAC GGGCTGGATC AGGCCATCAC GCCCGATGGC | 360 |
| CAGGGCGGCG GGCAGATCGG CGATAATCCT TTA CTGAAAG CCATGCTGAA GCTTATTGCA | 420 |
| CGCATGATGG ACGGCCAAAG CGATCAGTTT GGCCAACCTG GTACGGGCAA CAACAGTGCC | 480 |
| TCTTCCGGTA CTTCTTCATC TGGCGGTTCC CCTTTTAAAG ATCTATCAGG GGGGAAGGCC | 540 |
| CCTTCCGGCA ACTCCCCTTC CGGCAACTAC TCTCCCGTCA GTACCTTCTC ACCCCCATCC | 600 |
| ACGCCAACGT CCCCTACCTC ACCGCTTGAT TTCCCTTCTT CTCCCACCAA AGCAGCCGGG | 660 |
| GGCAGCACGC CGGTAACCGA TCATCCTGAC CCTGTTGGTA GCGCGGGCAT CGGGGCCGGA | 720 |
| AATTCGGTGG CCTTCACCAG CGCCGGCGCT AATCAGACGG TGCTGCATGA CACCATTACC | 780 |
| GTGAAAGCGG GTCAGGTGTT TGATGGCAAA GGACAAACCT TCACCGCCGG TTCAGAATTA | 840 |
| GGCGATGGCG GCCAGTCTGA AAACCAGAAA CCGCTGTTTA TACTGGAAGA CGGTGCCAGC | 900 |
| CTGAAAAACG TCACCATGGG CGACGACGGG GCGGATGGTA TTCATCTTTA CGGTGATGCC | 960 |
| AAAATAGACA ATCTGCACGT CACCAACGTG GGTGAGGACG CGATTACCGT TAAGCCAAAC | 1020 |
| AGCGCGGGCA AAAAATCCCA CGTTGAAATC ACTAACAGTT CCTTCGAGCA CGCCTCTGAC | 1080 |
| AAGATCCTGC AGCTGAATGC CGATACTAAC CTGAGCGTTG ACAACGTGAA GGCCAAAGAC | 1140 |
| TTTGGTACTT TTGTACGCAC TAACGGCGGT CAACAGGGTA ACTGGGATCT GAATCTGAGC | 1200 |
| CATATCAGCG CAGAAGACGG TAAGTTCTCG TTCGTTAAAA GCGATAGCGA GGGGCTAAAC | 1260 |
| GTCAATACCA GTGATATCTC ACTGGGTGAT GTTGAAACC ACTACAAAGT GCCGATGTCC | 1320 |
| GCCAACCTGA AGGTGGCTGA ATGA | 1344 |

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 447 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

| | | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Ser | Ile | Leu | Thr | Leu | Asn | Asn | Asn | Thr | Ser | Ser | Ser | Pro | Gly | Leu | 1 | 5 | 10 | 15 |
| Phe | Gln | Ser | Gly | Gly | Asp | Asn | Gly | Leu | Gly | Gly | His | Asn | Ala | Asn | Ser | 20 | 25 | 30 | |
| Ala | Leu | Gly | Gln | Gln | Pro | Ile | Asp | Arg | Gln | Thr | Ile | Glu | Gln | Met | Ala | 35 | 40 | 45 | |
| Gln | Leu | Leu | Ala | Glu | Leu | Leu | Lys | Ser | Leu | Leu | Ser | Pro | Gln | Ser | Gly | 50 | 55 | 60 | |
| Asn | Ala | Ala | Thr | Gly | Ala | Gly | Gly | Asn | Asp | Gln | Thr | Thr | Gly | Val | Gly | 65 | 70 | 75 | 80 |
| Asn | Ala | Gly | Gly | Leu | Asn | Gly | Arg | Lys | Gly | Thr | Ala | Gly | Thr | Thr | Pro | 85 | 90 | 95 | |
| Gln | Ser | Asp | Ser | Gln | Asn | Met | Leu | Ser | Glu | Met | Gly | Asn | Asn | Gly | Leu | 100 | 105 | 110 | |
| Asp | Gln | Ala | Ile | Thr | Pro | Asp | Gly | Gln | Gly | Gly | Gly | Gln | Ile | Gly | Asp | 115 | 120 | 125 | |
| Asn | Pro | Leu | Leu | Lys | Ala | Met | Leu | Lys | Leu | Ile | Ala | Arg | Met | Met | Asp | 130 | 135 | 140 | |
| Gly | Gln | Ser | Asp | Gln | Phe | Gly | Gln | Pro | Gly | Thr | Gly | Asn | Asn | Ser | Ala | 145 | 150 | 155 | 160 |
| Ser | Ser | Gly | Thr | Ser | Ser | Ser | Gly | Gly | Ser | Pro | Phe | Asn | Asp | Leu | Ser | 165 | 170 | 175 | |
| Gly | Gly | Lys | Ala | Pro | Ser | Gly | Asn | Ser | Pro | Ser | Gly | Asn | Tyr | Ser | Pro | 180 | 185 | 190 | |
| Val | Ser | Thr | Phe | Ser | Pro | Pro | Ser | Thr | Pro | Thr | Ser | Pro | Thr | Ser | Pro | 195 | 200 | 205 | |
| Leu | Asp | Phe | Pro | Ser | Ser | Pro | Thr | Lys | Ala | Ala | Gly | Gly | Ser | Thr | Pro | 210 | 215 | 220 | |
| Val | Thr | Asp | His | Pro | Asp | Pro | Val | Gly | Ser | Ala | Gly | Ile | Gly | Ala | Gly | 225 | 230 | 235 | 240 |
| Asn | Ser | Val | Ala | Phe | Thr | Ser | Ala | Gly | Ala | Asn | Gln | Thr | Val | Leu | His | 245 | 250 | 255 | |
| Asp | Thr | Ile | Thr | Val | Lys | Ala | Gly | Gln | Val | Phe | Asp | Gly | Lys | Gly | Gln | 260 | 265 | 270 | |

Thr Phe Thr Ala Gly Ser Glu Leu Gly Asp Gly Gly Gln Ser Glu Asn
 275 280 285
 Gln Lys Pro Leu Phe Ile Leu Glu Asp Gly Ala Ser Leu Lys Asn Val
 290 295 300
 Thr Met Gly Asp Asp Gly Ala Asp Gly Ile His Leu Tyr Gly Asp Ala
 305 310 315 320
 Lys Ile Asp Asn Leu His Val Thr Asn Val Gly Glu Asp Ala Ile Thr
 325 330 335
 Val Lys Pro Asn Ser Ala Gly Lys Lys Ser His Val Glu Ile Thr Asn
 340 345 350
 Ser Ser Phe Glu His Ala Ser Asp Lys Ile Leu Gln Leu Asn Ala Asp
 355 360 365
 Thr Asn Leu Ser Val Asp Asn Val Lys Ala Lys Asp Phe Gly Thr Phe
 370 375 380
 Val Arg Thr Asn Gly Gly Gln Gln Gly Asn Trp Asp Leu Asn Leu Ser
 385 390 395 400
 His Ile Ser Ala Glu Asp Gly Lys Phe Ser Phe Val Lys Ser Asp Ser
 405 410 415
 Glu Gly Leu Asn Val Asn Thr Ser Asp Ile Ser Leu Gly Asp Val Glu
 420 425 430
 Asn His Tyr Lys Val Pro Met Ser Ala Asn Leu Lys Val Ala Glu
 435 440 445

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGGAACCNNN NCNNNNNNNN NNCCACTCAA T